Fate of soluble methionine in African trypanosomes: effects of metabolic inhibitors

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The metabolism of [³⁵S]methionine in cultured bloodstream forms of African trypanosomes was followed using flow-through radiodetection linked to liquid chromatography separation. The effects of a transmethylation inhibitor, sinefungin, and of the ornithine decarboxylase inhibitor, DL- α -difluoromethylornithine (Ornidyl; DFMO), on methionine metabolism were also observed. Trypanosomes rapidly incorporated [³⁵S]methionine into S-adenosylmethionine (AdoMet) and the metabolites methylthioadenosine, S-adenosylhomocysteine, homocysteine, cystathionine, cysteine and glutathione. Untreated trypanosomes excreted large quantities of cystathionine and cysteine into the growth medium. DFMO-treated cells formed larger quantities of AdoMet more rapidly than did control cells, as was evident from initial time points (30 min and 1 h). Decarboxylated AdoMet,

present in trace quantities in control cells, accumulated in DFMO-treated cells. Sinefungin increased the AdoMet concentrations ~20-fold over that of controls after a 6 h incubation with [³⁵S]methionine, while cystathionine and cysteine levels decreased. The half-life ($t_{1/2}$) and rate of turnover of AdoMet were measured in cells treated with DFMO or sinefungin. DFMO treatment caused a substantial increase in the rate of AdoMet utilization, while sinefungin extended the $t_{1/2}$ and lowered AdoMet turnover. These studies show that trypanosomes rapidly metabolize methionine through AdoMet to intermediates of the polyamine and transmethylation pathways. Agents inhibiting these pathways rapidly affect the concentration and rate of utilization of AdoMet, significantly changing the concentrations of metabolites.

INTRODUCTION

S-Adenosylmethionine (AdoMet) is synthesized from adenosine and ATP and is essential for two fundamental cellular events: as an aminopropyl group donor in the formation of the polyamines spermidine and spermine, and as a methyl group donor in the addition of ethyl groups to cell components. In contrast to mammalian cells, little is known concerning the formation and fate of AdoMet in African trypanosomes. Stimulating this study, however, are the findings that AdoMet levels increase nearly 50-fold in trypanosomes treated *in vivo* with the ornithine decarboxylase (ODC) inhibitor DL- α -difluoromethylornithine (Ornidyl; DFMO) [1] and that this increase is the result of an AdoMet synthetase that is not sensitive to inhibition by its product [2]. Mammalian cells treated with DFMO show increases in AdoMet of 2-fold or less [3,4]. Of related interest is the flow of sulphur-containing metabolites through S-adenosylhomocysteine (AdoHcy), the product of transmethylation reactions (Figure 1). AdoHcy is toxic if left to accumulate in cells, and is rapidly cleaved by its hydrolase [5]. The resulting adenosine is recycled in mammalian cells through purine salvage, whereas homocysteine can be remethylated to methionine or transsulphurated to glutathione, cysteine and other sulphur-containing intermediates [5] (Figure 1).

In this study we have examined the fate of [³⁵S]methionine in bloodstream trypanosomes under normal growth conditions and in the presence of agents affecting AdoMet metabolism: sinefungin, a trypanocidal analogue of AdoMet and an inhibitor of transmethylation reactions [6–8]; and DFMO, which is used clinically against West African sleeping sickness [9,10]. These

agents, by interfering with methionine interconversion or transmethylation in bloodstream trypanosomes, could alter the availability of methionine and consequently rates of AdoMet synthesis or utilization.

MATERIALS AND METHODS

Organisms

Bloodstream forms of *Trypanosoma brucei brucei* LAB 110 EATRO were grown continuously at 37 °C in modified Iscove's modified Dulbecco's medium containing 20% (v/v) horse serum (HMI-18 medium) [11]. Stock cultures were grown in 3% CO₂/97% air in 24-well plates, using 1 ml of medium per well and replacing 0.5 ml of the medium daily. Cultures routinely reached $(0.5\text{--}1.0) \times 10^7$ cells/ml.

Experimental design

Cells were grown to a density of $(0.5\text{--}1.0) \times 10^7$ cells/ml in 750 ml tissue culture flasks (Falcon, Oxnard, CA, U.S.A.), harvested by centrifugation (3000 g; 10 min) and resuspended at 5×10^6 cells/ml in 20 ml of fresh HMI-18 medium. Populations to be drug-treated were preincubated for 4 h with IC₅₀ values of inhibitors that had been predetermined from *in vitro* growth curves. These were 0.5 mM DFMO and 2 μ M sinefungin. After preincubation, cells were resuspended (20 ml/flask) in fresh HMI-18 without methionine, but containing 200 μ Ci (1.7 nmol) of [³⁵S]methionine (1175 Ci/mmol; E.I. DuPont, NEN Products, Boston, MA, U.S.A.) and the appropriate drug. At intervals up

Abbreviations used: DFMO, DL- α -difluoromethylornithine; AdoMet, S-adenosylmethionine; MTA, 5'-methylthioadenosine; AdoHcy, S-adenosylhomocysteine; ODC, ornithine decarboxylase; dAdoMet, decarboxylated AdoMet; RT, retention time.

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Figure 1 Metabolism of methionine in trypanosomes

Numbers represent enzyme reactions or sequences: 1, AdoMet synthase; 2, transmethylation reactions; 3, AdoHcy hydrolase; 4, methylfolate:homocysteine methyltransferase; 5, AdoMet decarboxylase; 6, spermidine synthase; 7, MTA phosphorylase; 8, regeneration of methionine from methylthioribose 1-phosphate; 9, cystathionine synthase; 10, cystathionase; 11, glutathione synthesis; 12, trypanothione synthesis.

to 6 h, cells in individual flasks were harvested by centrifugation (3000 g; 10 min), washed with HMI-18 minus methionine and extracted overnight at 0–4 °C with 250 µl of 10% (v/v) trichloroacetic acid [approx. $(1.0\text{--}1.5) \times 10^8$ cells/flask]. The precipitate was centrifuged and extracts were frozen at –75 °C and assayed within 1 week.

HPLC analysis

The overall methodology has been described previously [1,12]. HPLC analysis was done using a Perkin–Elmer Series 410 liquid chromatograph with a Rheodyne 50 µl loop and injector (North American Instrument Division, New Hyde Park, NY, U.S.A.). IN/US 'In Flow ES' served as the scintillant in a 3:1 (v/v) scintillant/solvent ratio. A Perkin–Elmer C-18 Percosil column (10 µm) was the solid phase. Detection and quantification of radiolabel were carried out using an IN/US flow-through radio-detector and B-RAM integration software (Program 1.4; IN/US Systems, Tampa, FL, U.S.A.). A Perkin–Elmer LC-95 UV–visible absorbance detector was connected in tandem with the radio-detector. The mobile phases consisted of: solvent A, 980 ml of 0.1 M NaH_2PO_4 containing 8 mM octanesulphonic acid (pH 2.65 with H_3PO_4); solvent B, 740 ml of 150 mM NaH_2PO_4 , 260 ml of acetonitrile and 8 mM octanesulphonic acid (pH 3.25 with H_3PO_4). The gradient system previously used was modified from that of Yarlott and Bacchi [1]. A linear gradient (1 ml/min) was used, starting with an isocratic step of 100% solvent A for 8 min, and leading to 100% solvent B in 30 min. This was followed by an isocratic step of 100% B for 5 min before regenerating the starting conditions. Radiolabelled standards were [^{35}S]-

methionine [retention time (RT): 11:00 min] and *S*-adenosyl-L-[methyl- ^{14}C]methionine (RT) 31:30). Standards, detected by UV spectrometry (254 nm), were (with RTs given in min:s, in parentheses): cysteine (3:50), cystathionine (4:45) glutathione (reduced; 5:30), homocysteine (14:30), oxidized glutathione (17:40), AdoHcy (29:30), reduced trypanothione (bis-glutathionylspermidine [13]; 30:30), methylthioadenosine (MTA; 34:00) and decarboxylated AdoMet (dAdoMet; 38:00). Methionine sulfoxide was a minor (5–12%) contaminant of the [^{35}S]methionine with an RT of 4:00 min. All data involving cysteine formation have been corrected to reflect this. Because cysteine and cystathionine eluted close to one another, not all integrations fully separated them, and they are reported as a combined total.

Chemicals

DFMO was a gift from the Marion Merrell Dow Research Institute (Cincinnati, OH, U.S.A.). dAdoMet, used as an HPLC standard, was kindly donated by Dr. Carl Porter (Roswell Park Cancer Institute, Buffalo, NY, U.S.A.). Trypanothione was purchased from Bachem (Bubendorf, Switzerland). All other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile was spectral grade from Fisher Scientific (Springfield, NJ, U.S.A.).

RESULTS

Interconversion of [^{35}S]methionine

Control (untreated) populations of trypanosomes rapidly assimilated [^{35}S]methionine and converted it into AdoMet,

Figure 2 HPLC traces of trypanosome extracts showing distribution of ^{35}S -labelled metabolites

Cells were incubated for the times shown with $10\ \mu\text{Ci/ml}$ [^{35}S]methionine in methionine-free HMI-18, and trichloroacetic acid extracts were analysed as described in the Materials and methods section. (a) Control cells; (b) cells pretreated for 4 h with $2\ \mu\text{M}$ sinefungin. Traces are representative of at least three separate experiments. Abbreviation: Cyst., cystathionine.

AdoHcy and related products. In a series of experiments in which control, untreated trypanosomes were incubated for up to 6 h in methionine-free HMI-18 plus [^{35}S]methionine, label was initially (1 h) incorporated into cystathionine, cysteine, glutathione, AdoHcy and AdoMet (Figure 2a). Trace amounts of dAdoMet and MTA also appeared. Since samples were not maintained in the reduced state, reduced trypanothione was not detected. Quantification of a chromatogram from a typical experiment is presented in Figure 3(a).

A decrease in the methionine peak was evident by 2 h, and increases in the levels of most metabolites were evident. AdoMet, glutathione and homocysteine peaked at 4 h, and by 6 h all metabolite levels had begun to decline (Figures 2a and 3a).

The distribution of ^{35}S -labelled metabolites after a 4 h incubation of control cells (three experiments) is summarized in

Table 1. Total uptake of [^{35}S]methionine into the soluble fraction peaked at 4 h at $8.5\text{--}12.7\ \text{fmol}/5 \times 10^8$ cells, with AdoMet and related intermediates accounting for $5.9\text{--}9.7\ \text{fmol}/5 \times 10^8$ cells, or 72–77 % of the total ^{35}S in the soluble fraction (Table 1). On the basis of cell protein content (3×10^8 trypanosomes/mg of protein), the total soluble ^{35}S incorporation at 4 h ranged from approx. 0.4 to 0.6 pmol/mg of protein.

AdoMet levels ranged from 21 to 43 % of the total [^{35}S]methionine incorporated in the soluble fraction, indicating that there was a rapid and considerable conversion of methionine to AdoMet. In contrast, AdoHcy levels were low throughout the incubations, peaking at 2–4 h (Figure 3a) and representing 5–7 % of total incorporated label (Table 1), an apparent indication of the rapidity with which AdoHcy is hydrolysed. AdoMet/AdoHcy ratios can provide a rough index of the transmethylation potential

Figure 3 Quantification of ^{35}S -labelled intermediates in trypanosome cell extracts: effects of sinefungin

Data are from Figure 2. (a) Control cells; (b) cell treated for 4 h with $2\ \mu\text{M}$ sinefungin. ▲, Methionine; ▼, AdoMet; ●, cystathionine + cysteine; ●, homocysteine; ◆, AdoHcy; ■, glutathione.

of a cell [14]. In the present studies this ratio ranged from 2:1 to 9:1, indicating that normal methylation was occurring [14]. Likewise, in control cells for all experiments, homocysteine formed only a minor (10% or less; Table 1) fraction of the ^{35}S incorporated, indicative of its further metabolism.

Significant ^{35}S accumulated in the trans-sulphuration intermediates cysteine, cystathionine and GSH, accounting for 19–37% of the total soluble label. The rapidity with which label was incorporated into cystathionine and cysteine (Figure 2a) and the presence of only low (<1%) amounts of dAdoMet and MTA (Table 1), intermediates in polyamine synthesis, are also indicative of a substantial flow of metabolites from AdoMet through AdoHcy. Analysis of the incubation medium to determine the nature and quantity of excreted metabolites revealed that $1.17 \pm 0.44\ \text{pmol}$ of cysteine + cystathionine/ml of medium

was released over a 4 h incubation period ($n = 4$). This level was 100 times the total ^{35}S found in the cell at the 4 h time point of incubation, and is indicative of a high rate of transmethylation followed by entry of the homocysteine into the trans-sulphuration pathway. An indication of the relative use of methionine for transmethylation in trypanosomes can be obtained by the finding that [^{35}S]methionine was incorporated into protein at approx. $80\ \text{pmol}/5 \times 10^6\ \text{cells}$ ($400\ \text{pmol}/\text{mg}$ of protein), while [$1\text{-}^{14}\text{C}$]methionine was incorporated at >4–6 times this level ($300\text{--}500\ \text{pmol}/5 \times 10^6\ \text{cells}$) as result of transmethylation (B. Goldberg, N. Yarlett and C. Bacchi, unpublished work).

Effects of antimetabolites on metabolism of [^{35}S]methionine

During the course of our studies on the effects of DFMO on African trypanosomes, it became apparent that the total blockade of polyamine synthesis was only a part of the effect of DFMO on these parasites: trypanosome AdoMet and dAdoMet increased >50-fold and >300-fold respectively over 36 h of treatment in model infections [1,2]. Studies with an inhibitor of AdoMet decarboxylase (MDL73811) also indicated that a rapid elevation in the AdoMet concentration occurred [4]. To examine the effects of inhibition of polyamine synthesis on the incorporation of [^{35}S]methionine, we pretreated cells with DFMO for 4 h prior to incubation with label, then examined the fate of the label over the course of 4–6 h incubations.

Cells pretreated with $500\ \mu\text{M}$ DFMO rapidly assimilated [^{35}S]methionine into AdoMet, increasing levels of this metabolite to 2.5-fold those of controls at 30 min (Figure 4); AdoMet remained elevated for the duration of the 4 h study. dAdoMet gradually increased in DFMO-treated cells to $0.37\ \text{fmol}/5 \times 10^6\ \text{cells}$, about 20% of the AdoMet level, after 4 h (Figure 4).

dAdoMet was detectable only in trace amounts in control cells. The trans-sulphuration products cystathionine and cysteine increased in control cells to about twice the levels in DFMO-treated cells, while AdoHcy, homocysteine and glutathione levels remained about the same in both sets of cells. Levels of methionine were also similar. At 4 h, AdoMet made up 29% of the total [^{35}S] incorporated into the soluble fraction of control cells and 41% of [^{35}S] incorporation in DFMO-treated cells. If dAdoMet was considered as part of the AdoMet pool for DFMO-treated cells, then 50% of the label was found in this intermediate. Pools of the trans-sulphuration intermediates cysteine and cystathionine also contained a substantial portion of the label: 25% in DFMO-treated cells and 46% in control cells.

Examination of supernatant media from this experiment indicated that at 4 h control cells had released $1.19\ \text{pmol}$ of cysteine + cystathionine/ml of medium, while DFMO-treated cells had released $5.81\ \text{pmol}/\text{ml}$. These data indicate that a significant level of transmethylation occurs on trypanosomes, and that the major byproducts of transmethylation, cysteine and cystathionine, are increased about 5-fold by treatment with DFMO.

The AdoMet analogue and inhibitor of protein transmethylation, sinefungin, is trypanocidal [6,8] and was examined for activity in the trypanosome incorporation system in a 6 h incubation study (Figures 2 and 3). Cells during this incubation remained motile and no lysis was observed, as checked by cell counts. Data at 4 h indicated that, for control cells, 21% of ^{35}S incorporated into the soluble fraction ($1.07\ \text{fmol}/5 \times 10^6\ \text{cells}$) appeared as AdoMet and 41% ($1.88\ \text{fmol}/5 \times 10^6\ \text{cells}$) as cysteine + cystathionine. Sinefungin-treated cells converted 45% ($2.72\ \text{fmol}$) to AdoMet and 33% ($2.01\ \text{fmol}$) to cysteine + cystathionine. The AdoMet value for treated cells was 2.5 times

Table 1 Distribution of [^{35}S]methionine in trichloroacetic acid-soluble fraction after a 4 h incubation with control (untreated) trypanosomesData are expressed as fmol/ 5×10^6 cells and as percentages of total label. RTs are given in min:s.

Compound	RT	Experiment 1		Experiment 2		Experiment 3	
		(fmol)	(%)	(fmol)	(%)	(fmol)	(%)
Cysteine	3:50	1.88	23.0	1.50	12	2.30	18
Cystathionine	4:45					2.40	19
GSH	5:30	1.18	14.3	0.90	7.3	—	—
Methionine	11:02	1.00	12.0	1.97	16	2.10	18
Homocysteine	14:30	0.87	10.5	0.30	2.4	0.85	6.7
GSSG	17:40	0.04	0.5	0.03	< 0.01	0.09	< 0.01
AdoHcy	29:30	0.50	6.0	0.60	4.8	0.92	7.3
AdoMet	31:30	1.37	23.0	5.50	44.7	2.73	21.6
MTA	34:00	0.05	0.6	0.02	< 0.01	—	—
dAdoMet	38:00	0.06	0.7	0.01	< 0.01	0.45	3.6
Unknown	—	1.56	18.3	1.53	12	0.76	6
Total soluble label	8.51			12.7		12.6	
Total ^{35}S -labelled intermediates (exclusive of methionine or unknown)	5.95			9.1		9.74	
Incorporation into intermediates (%)		5.95/8.21 = 72.5%		9.1/12.7 = 71.6%		9.74/12.6 = 77.3%	

that of controls at 4 h. However, in contrast to control cells, the AdoMet level in treated cells continued to rise, and at 6 h was nearly 20 times that of the control cells (9.5 versus 0.5 fmol; Figure 2). Incorporation of [^{35}S]methionine at this time point was 3.0 fmol/ 5×10^6 cells for controls and 14.2 fmol/ 5×10^6 cells for sinefungin-treated cells (i.e. nearly five times that of the controls). Interestingly, at 6 h, cystathionine + cysteine levels in sinefungin-treated cells had decreased to about one-third of the levels at 4 h. Subsequent experiments with sinefungin have indicated similar increases in AdoMet levels and total incorporation for treated cells.

AdoMet half-life

In parallel experiments, the half-life ($t_{1/2}$) of AdoMet in blood-form trypanosomes was determined in control, DFMO-treated and sinefungin-treated cells. Trypanosomes were preincubated with no addition, with DFMO (500 μM) or with sinefungin (2 μM) for 4 h in complete HMI-18 as described in the Materials and methods section, resuspended in methionine-free medium (plus appropriate inhibitors) with 40 μCi of [^{35}S]methionine for 1 h, and again resuspended in complete HMI-18. Samples were removed at intervals up to 4 h and the cell pellets were extracted with 10% (w/v) trichloroacetic acid and analysed by HPLC. The half-life of the [^{35}S]AdoMet formed was then obtained using linear regression analysis (Sigma Plot; Jandel Scientific) applied to the decline in AdoMet levels over a 2 or 4 h period. A typical experiment is shown in Figure 5. In Table 2 average values for two experiments are given. Control cells had a $t_{1/2}$ of 70 min and a turnover rate of 0.72 fmol/h per 5×10^6 cells. DFMO-treated cells initially exhibited a high concentration of AdoMet, about three times that of controls (Figure 5). The $t_{1/2}$ for AdoMet was 58 min and the turnover was 2.4 fpmol/h per 5×10^6 cells (Table 2). Sinefungin-treated cells had a long $t_{1/2}$ (138 min) and a turnover rate (0.26 fmol/h per 5×10^6 cells) one-third that of controls, indicating that two-thirds of AdoMet turnover, or approx. 0.5 fmol/h per 5×10^6 cells, could be ascribed to transmethylation events (control AdoMet turnover minus sinefungin AdoMet turnover). In a recent study with *Leishmania* spp. sinefungin was

found to inhibit 60% of total methylation of lipids and protein [15], and our values appear to be in good agreement with this.

DISCUSSION

Two considerations provided the impetus for this work: African trypanosomes are important parasites of humans and domesticated animals, causing human disease and economic hardship; and these organisms undergo a complex life cycle during which biochemical and morphological changes occur rapidly as the parasites alternate between vertebrate host and insect vector. The finding that antagonists of polyamine metabolism such as DFMO and MDL73811 were curative in vertebrate infections [16,17], and that one biochemical consequence of blocking polyamine synthesis was the significant accumulation of AdoMet [1,4], indicated that this aspect of trypanosome metabolism differed from that of the host. A central aspect of this view is the unregulated nature of trypanosome AdoMet synthase, which allows accumulation of this metabolite under conditions in which the pathways utilizing it are blocked [2]. In contrast, two of the three mammalian AdoMet synthase isoenzymes are inhibited by excess AdoMet, with liver (isoenzyme III) the only tissue capable of overproducing AdoMet [5].

The major metabolites of [^{35}S]methionine identified in the present study reflect those found in mammalian cells [5,18,19]. Control, untreated trypanosomes rapidly process methionine through AdoMet, AdoHcy, homocysteine and the trans-sulphuration products cystathionine and cysteine, as well as the metabolite glutathione. AdoMet was a major metabolite, accounting for 24–45% of the total soluble ^{35}S in the three experiments shown. This compares to 20–23% found for [^{35}S]methionine incorporation in human lymphoid cells [20]. This value was obtained by measuring accumulation of homocysteine in the medium and is therefore due to transmethylation only. Comparisons of the fate of [^{35}S]methionine in trypanosomes with that in other cell systems are less direct: AdoMet made up only 3.9% of the total [^{35}S]methionine incorporated into *Leishmania* spp. promastigotes after overnight incubation [21]. In the present

Figure 4 Quantification of intermediates of [³⁵S]methionine in trypanosome cell extracts: effects of DFMO

Cells were incubated in methionine-free HMI-18 with 10 μCi/ml [³⁵S]methionine, and trichloroacetic acid extracts were analysed as described in the Materials and methods section. (a) Control cells; (b) cells were pretreated for 4 h with 500 μM DFMO. ▲, Methionine; ▼, AdoMet; ●, cystathionine + cysteine; ●, homocysteine; ◆, AdoHcy; ■, glutathione; ▽, dAdoMet.

work, however, AdoMet levels peaked at 4 h, and were considerably lower thereafter.

Release of cystathionine and cysteine into the medium was not expected, although this might have been predicted from the rapid build-up and processing of AdoMet. The mean level of intermediates in the medium from control cells was 1.17 pmol/5 × 10⁶ cells, i.e. about 10 times the level of ³⁵S intermediates found inside control cells at the time of peak incorporation (Table 1). In many mammalian cells, homocysteine is the basis for regeneration of methionine through methylfolate:homocysteine methyltransferase (EC 2.1.1.13) [5]. Although this enzyme is active in trypanosome extracts [1] and presumably can regenerate meth-

Figure 5 Utilization of [³⁵S]AdoMet in trypanosomes treated with DFMO or sinefungin

Trypanosomes were grown in methionine-depleted HMI-18 containing 10 μCi/ml [³⁵S]-methionine for 1 h, washed and resuspended in methionine-replete HMI-18. Samples were taken at the indicated time points and trichloroacetic acid samples were analysed as described. ▲, Pretreated for 4 h with 500 μM DFMO; ■, pretreated for 4 h with 2 μM sinefungin; ●, control. Traces are of a representative experiment, and were subjected to regression analysis (Sigma Plot).

Table 2 *t*_{1/2} and turnover rate of AdoMet in trypanosomes: effect of DFMO and sinefungin

Trypanosomes were preincubated for 4 h with DFMO (500 μM) or sinefungin (2 μM) in complete HMI-18. They were then washed and resuspended in HMI-18 minus methionine, with appropriate drugs and 40 μCi of [³⁵S]methionine, for 1 h and again resuspended in complete HMI-18. Aliquots were removed at intervals, extracted with trichloroacetic acid and analysed by HPLC. Data are the averages from two experiments. Slopes for turnover rate and *t*_{1/2} were obtained by linear regression analysis.

	<i>t</i> _{1/2} of AdoMet (min)	Turnover (fmol/5 × 10 ⁶ cells)
Control	70	0.72
DFMO	58	2.40
Sinefungin	138	0.26

ionine, the present series of experiments could not determine the degree to which remethylation of homocysteine is operative.

One of the critical features of AdoMet metabolism and transmethylation events is the need for the rapid removal of AdoHcy, the toxic byproduct of methyl group transfer [18]. In the present study, AdoHcy levels never exceeded 10% of the total soluble ³⁵S incorporated, even in the presence of DFMO, which blocks AdoMet use in polyamine synthesis and causes its accumulation [1], and diversion to methylation events (B. Goldberg, N. Yarlett and C. Bacchi, unpublished work). This would indicate that trypanosome AdoHcy hydrolase, present in trypanosome extracts [1], is sufficiently active *in situ* to handle increases in transmethylation activity [5, 18]. Homocysteine levels are also < 10% of the total, indicating rapid removal via remethylation or trans-sulphuration.

The most conspicuous effects of pretreatment with DMFO or sinefungin on [35 S]methionine metabolism in trypanosomes concerned AdoMet synthesis. DFMO-treated cells had initially (30 min) elevated levels of AdoMet, 3.5-fold above that of controls (Figure 4), but by 4 h, both levels were similar. DFMO treatment also resulted in a 5-fold increase in cystathionine and cysteine levels in the medium. The increase in extracellular metabolites, rather than a change in the internal AdoMet level, may be the more significant indicator of increased flow through AdoMet and transmethylation. In support of this, intracellular cystathionine and cysteine levels were not sharply different in control and DFMO-treated cells. In contrast, AdoMet levels in cells treated with sinefungin increased steadily over the 6 h incubation with [35 S]methionine, reaching nearly 20 times control levels. At this time intracellular cystathionine and cysteine levels were decreased 70% below controls (Figure 3), an additional indication that transmethylation was lowered. In trypanosomes, sinefungin is a potent inhibitor of protein methylase II (K_i 1.6 μ M) and affects protein methylases I and III to a lesser extent ([7]; N. Yarlett and C. Bacchi, unpublished work). In *Leishmania* spp., sinefungin lowered incorporation of S-[3 H]-methyl-AdoMet into proteins and lipids by about 60% [15], and caused morphological changes which were antagonized by AdoMet [22]. However, [35 S]AdoMet levels in *Leishmania* barely doubled after overnight incubation with sinefungin and [35 S]methionine, indicating that AdoMet synthase in these organisms may be more closely regulated than in trypanosomes [21].

Half-life calculations of AdoMet levels revealed a $t_{1/2}$ of 70 min for control cells (Table 2). This compares with 7.5 min obtained with Friend erythroleukaemia cells in pulse-chase experiments with [35 S]methionine [23] and 2 h for *Leishmania* spp. promastigotes incubated with [methyl- 3 H]AdoMet [15]. Although the present estimate for trypanosomes does not account for a component of AdoMet synthesis due to the presence of [35 S]methionine in washed cells, the concentration of label was no greater than 25% of total [35 S]AdoMet concentration at the start of the time period from which the $t_{1/2}$ was calculated. An indication of the changes in trypanosome metabolism as a result of interference with AdoMet metabolism is given by the alteration of AdoMet utilization: control cells utilized AdoMet at the rate of 0.72 fmol/h per 5×10^6 cells, while DFMO-treated cells, maintaining a similar $t_{1/2}$ (58 min), utilized AdoMet at 2.4 fmol/h per 5×10^6 cells. Thus, although the $t_{1/2}$ values are similar in the two groups of cells, a substantial increase in turnover of AdoMet occurs in DFMO-treated cells. A related study, examining protein methylation in DFMO-treated trypanosomes, indicated a > 10-fold increase in methylation in treated cells (B. Goldberg, N. Yarlett and C. Bacchi, unpublished work). Ultimately, with prolonged exposure to DFMO *in situ*, AdoMet synthesis must exceed the rate of methylation, since after 36 h of exposure trypanosomes had accumulated 35.9 pmol/mg of protein for an internal concentration of > 1 mM [1,2]. In the present study DFMO-treated cells also accumulated dAdoMet at a rate of 5–10% of that of AdoMet, but dAdoMet slowly declined over the 2–4 h time points. ($t_{1/2}$ = 60 min). This may signify that over this time of exposure of DFMO (4–10 h), a low level of intracellular putrescine remains and the dAdoMet is slowly utilized in spermidine synthesis. In trypanosomes incubated *in*

vitro with 500 μ M DFMO for 1 h, inhibition of ODC activity was nearly 100% (C. Bacchi, unpublished work).

The extended $t_{1/2}$ (138 min) and low turnover rate obtained with sinefungin-treated trypanosomes are in keeping with the potent inhibition of protein methylation by sinefungin in haemoflagellates. Moreover, the accumulation of AdoMet by sinefungin-treated cells is an indication of the normally rapid utilization of AdoMet by trypanosomes.

Overall, the actions of DFMO and sinefungin are apparent in their disruption of AdoMet metabolism. DFMO caused an overall increase in AdoMet utilization, coupled with elevated excretion of cysteine and cystathionine into the medium. A parallel effect was seen in livers of rats fed excess methionine, which resulted in significant elevation of trans-sulphuration products including taurine, cysteine and glutathione [19].

The present study shows that trypanosome methionine metabolism is geared to rapid synthesis and utilization of AdoMet and that trans-sulphuration intermediates are key products of this metabolism. Additional studies are needed to determine the partitioning of AdoMet between polyamine synthesis and trans-methylation, and to determine the degree of remethylation of homocysteine to regenerate methionine.

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